REMARKS

Claims 123-126 and 128-131 presently appear in this case. No claims have been allowed. The official action of July 28, 2005, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention relates to a method for converting $CD38^{-/low}$ $CXCR4^{-/low}$ stem cells into $CD38^{-/low}$ $CXCR4^{+}$ stem cells. This conversion is accomplished by stimulating the starting stem cells for up to five days with a suitable agent capable of causing such conversion.

Claims 123-126 and 128-131 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Kanz in view of Mohle. The examiner states that the evidence previously provided to demonstrate "unexpected results" and thus non-obviousness is not commensurate in scope with the claims as written and that it is not evident from the declaration that the alleged unexpected properties are commensurate in scope with the breadth of the claims as written. The examiner further does not consider the post-filing evidence presented in the declaration as being relevant to the claims as written. The examiner states that Kanz teaches the stimulation of the cells with the growth factor for "up to 28 days," which range includes five days or fewer. Thus, the examiner considers that the recited range lies within the range taught by Kanz. This rejection is respectfully traversed.

It must be emphasized that the present invention addresses the issue of the lack of availability of suitable

stem cells for urgent transplantation. There has been a long felt need for methods for achieving suitable stem cells in a short time for urgent transplantation. That problem is solved by the methods of the present invention and yet it is not addressed by the prior art of record.

It must also be emphasized that the present rejection is one based on obviousness and not anticipation. Whether or not the CXCR4 marker was inherently increased within the first five days of the treatment of Kanz is irrelevant to an obviousness determination. In other words, that which is inherent is not necessarily obvious. See *In re Sporman*, 150 USPQ 449, 452 (CCPA 1966) ("The inherency of an advantage and its obviousness are entirely different questions. That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown."), quoted with approval in *In re Shetty*, 195 USPQ 753 (CCPA 1977). See also *In re Henderson*, 146 USPQ 372, 375 (CCPA 1965) ("The mere notion that such discovery 'would flow naturally' from what appellant did does not mean that the discovery could be predicted from what the art had done.")

Suitable stem cells for transplantation, according to the methods of the present invention, are stem cells obtained by stimulating the stem cells for a short time (five days or fewer) with suitable factors that induce exposure of CXCR4 on the surface of said cells. The inventor found that stem cells having more CXCR4 exposed on the cell surface, as compared to cells having normal or low levels of CXCR4 exposed

on the surface, show enhanced migration, adhesion to stroma cells, engraftment to the bone marrow and self renewal (Examples 3 and 4). Thus, the method of the invention allows an increase in the percentage of stem cells suitable for engraftment in a short time, a time that is not long enough to obtain cell expansion. Therefore, the number of cells before and after applying the method of the invention is about the same, only that the cells obtained after applying the method of the invention have more CXCR4 exposed on the cell surface and therefore are more suitable for transfection.

The suitable stem cells for transplantation according to Kanz are $CD34^+$ expanded cells. The entire purpose for stimulating cells with various factors in the Kanz patent is to cause the cells to expand, not to cause exposure of CXCR4 to be induced on the surface of cells that are not expanding.

The examiner states that the Kanz teaching of stimulation for "up to 28 days" includes five days or fewer. However, those of ordinary skill in the art aware of the publication of Kanz related to the experimentation in a Kanz patent, would understand that one would get no expansion in less than five days. Attached hereto is a copy of Brugger, Kanz et al, Blood 81(10):2579-2584 (1993). This publication shows that, indeed, expansion of $CD34^+$ cells does not occur up to six days of incubation of the $CD34^+$ cells with the preferred combination of factors described by Kanz. The publication states at page 2582, left column, lines 3-10:

> The number of clonogenic progenitor cells of different lineages started to increase at day 6 and peaked at days 12 to 14 in culture (Fig 5).

Therefore, the publication of Brugger et al teaches away from making the presently-claimed invention. Clearly there is no motivation to stop the stimulation of stem cells by day five or fewer in Kanz. Any *prima facie* case of obviousness indicated by the examiner has been overcome by Kanz' own evidence that expansion does not begin before day 6. Accordingly, there would have been no motivation whatsoever to stop the stimulation before day 6. Thus, one of ordinary skill in the art reading Kanz and Brugger would not understand that any changes would have been made to the cells by that time.

Accordingly, as the present claims require stimulating the stem cells "for up to five days" with the suitable agent and as no one of ordinary skill in the art reading Kanz in light of Brugger, either alone or in combination with Mohle, would have any motivation to stop the stimulation of Kanz by day 5, any *prima facie* case of obviousness obtained by the examiner has been overcome, and the present invention has been demonstrated to be unobvious. The stimulation of the appearance of CXCR4 on the surface of the cells by stimulation of fewer than five days is totally unexpected from any reading of the references of record and, thus, the step of stopping the stimulation within five days would not have been obvious from any combination of the

references of record. Reconsideration and withdrawal of this rejection are, therefore, respectfully urged.

Claim 123 has been slightly amended merely to improve the claim language in setting forth a positive method step of stimulating, rather than claiming the stimulation passively.

It is submitted that all of the claims now present in the case clearly define over the references of record and fully comply with 35 U.S.C. §112. Reconsideration and allowance are, therefore, earnestly solicited.

Respectfully submitted,

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Ex Vivo Expansion of Enriched Peripheral Blood CD34+ Progenitor Cells by Stem Cell Factor, Interleukin-1β (IL-1β), IL-6, IL-3, Interferon-γ, and Erythropoietin

By Wolfram Brugger, Wolfgang Möcklin, Shelly Heimfeld, Ronald J. Berenson, Roland Mertelsmann, and Lothar Kanz

To provide sufficient numbers of peripheral blood progenitor cells (PBPCs) for repetitive use after high-dose chemotherapy, we investigated the ability of hematopoietic growth factor combinations to expand the number of clonogenic PBPCs ex vivo. Chemotherapy plus granulocyte colony-stimulating factor (G-CSF) mobilized CD34+ cells from 18 patients with metastatic solid tumors or refractory lymphomas were cultured for up to 28 days in a liquid culture system. The effects of interleukin-1β (IL-1), IL-3, IL-6, granulocyte-macrophage–CSF (GM-CSF), G-CSF, macrophage-CSF (M-CSF), stem cell factor (SCF), erythropoietin (EPO), leukemia inhibitory factor (LIF), and interferon-γ, as well as 36 combinations of these factors were tested. A combination of five hematopoietic growth factors, including SCF, EPO, IL-1, IL-3, and IL-6, was identified as the optimal combination of growth factors for both the expansion of total nucleated cells as well as the expansion of clonogenic progenitor cells. Proliferation peaked at days 12 to 14, with a median 190-fold increase (range, 46- to 930-fold) of total clonogenic progenitor cells. Expanded progenitor cells generated myeloid (colony-forming unit–granulocyte-macrophage), erythroid (burst-forming unit-erythroid), as well as multilineage (colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte) colony-forming units. The number of multilineage colonies increased 250-fold (range, 33- to 589-fold) as compared with pre-expansion values. Moreover, the absolute number of early hematopoietic progenitor cells (CD34+/HLA-DR−; CD34+/CD38−), as well as the number of 4-HC–resistant progenitors within expanded cells increased significantly. Interferon-γ was shown to synergize with the 5-factor combination, whereas the addition of GM-CSF significantly decreased the number of total clonogenic progenitor cells. Large-scale expansion of PB CD34+ cells (starting cell number, 1.5 × 10⁶ CD34+ cells) in autologous plasma supplemented with the same 5-factor combination resulted in an equivalent expansion of progenitor cells as compared with the microculture system. In summary, our data indicate that chemotherapy plus G-CSF–mobilized PBPCs from cancer patients can be effectively expanded ex vivo. Moreover, our data suggest the feasibility of large-scale expansion of PBPCs, starting from small numbers of PB CD34+ cells. The number of cells expanded ex vivo might be sufficient for repetitive use after high-dose chemotherapy and might be candidate cells for therapeutic gene transfer.

PERIPHERAL BLOOD progenitor cells (PBPCs) are used with increasing frequency after high-dose chemotherapy to allow rapid, complete, and sustained hematopoietic reconstitution.[1-6] PBPCs can be mobilized after myelosuppressive chemotherapy,[7] upon administration of hematopoietic growth factors,[8-10] or after the combined administration of chemotherapy and growth factors.[3,9-11] Generally, three or more aphereses, processing at least 15 L of blood, are required to collect PBPCs in numbers sufficient for clinical use. To process smaller amounts of blood or to possibly avoid aphereses, we investigated the ability of different hematopoietic growth factor combinations to expand PBPCs ex vivo. This approach might provide sufficient numbers of clonogenic hematopoietic progenitor cells for repetitive clinical use after high-dose chemotherapy. In addition, ex vivo expanded PBPCs might be candidate cells for therapeutic gene transfer.[12] Moreover, because tumor cell contamination in hematopoietic stem cell collections is still a matter of debate,[13] ex vivo expansion of PBPCs might reduce the possibility of malignant cell contamination in disseminated cancer.[14]

Based on preclinical data showing synergistic effects of stem cell factor (SCF), interleukin-1β (IL-1), IL-3, and IL-6 on primitive hematopoietic progenitor cells in vitro,[15-19] we investigated combinations of growth factors including SCF as to their ability to induce the ex vivo expansion of chemotherapy plus granulocyte colony-stimulating factor (G-CSF)-mobilized PBPCs from patients undergoing PBPC transplantation. In addition, the requirements for large-scale expansion of PBPCs are reported.

MATERIALS AND METHODS

Study design. Eighteen consecutive patients undergoing PBPC transplantation were treated as part of their induction chemotherapy with conventional-dose VP16 (500 mg/m²), ifosfamide (4 g/m²), and cisplatin (50 mg/m²) (VIP) and subsequent administration of recombinant human G-CSF (Filgrastim; Amgen, Munich, Germany) at a dose of 5 μg/kg/d subcutaneously (SC) for 12 to 14 days to mobilize PBPCs.[11,20,21] Twelve patients with solid tumors and six patients with refractory non-Hodgkin's lymphoma were included; the median age of the study group was 41 years (range, 26 to 58 years). This study was approved by the local ethics committee and all patients gave informed consent. PBPCs were collected at days 10 through 12 after VIP chemotherapy as described previously.[20,21] CD34+ cells present within harvested PBPCs were enriched by immunoadsorption and subsequently expanded in suspension culture in the presence of a range of recombinant human growth factors and analyzed for their capacity to form clonogenic cells of different lineages in vitro. In addition, the phenotypic properties of expanded cells were studied by dual-color or triple-color flow cytometry. Large-scale expansion in the presence of the optimal combination of hematopoietic growth factors was performed in the latter two patients to investigate the feasibility of in vitro culture of PBPCs for their possible clinical application.

Positive selection of CD34+ cells from harvested PBPCs by immunoaffinity adsorption columns. Mononuclear cells (MNCs) from

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the apheresis product were isolated by density gradient centrifugation over Ficoll (1.077 g/dL; Pharmacia, Freiburg, Germany), washed twice in phosphate-buffered saline (PBS; Biochrom, Berlin, Germany), and adjusted to 2×10^8 MNCs/mL. MNCs were incubated with a biotinylated IgM anti-CD34 monoclonal antibody (MoAb) (clone 12.8), washed, and loaded onto an avidin-immunoaffinity column (Ceprate TM system; CellPro Inc, Bothell, WA), as described.[22] Adsorbed $CD34^+$ cells (target cell population) were removed from the avidin column and resuspended in RPMI 1640 medium (Seromed, Germany) supplemented with 3 mmol/L glutamine and 5×10^{-5} mol/L β-mercaptoethanol (both from Sigma, Deisenhofen, Germany). An aliquot of the $CD34^+$ target cell fraction was analyzed to assess purity and colony formation as described below. Unbound cells (nontarget cell fraction) were removed.

Expansion of enriched $CD34^+$ cells in suspension culture. Enriched $CD34^+$ cells were cultured in 96-well flatbottom microtiter plates (Falcon, Heidelberg, Germany) at 0.5 to 15×10^3/mL in supplemented RPMI 1640 medium containing 10% fetal calf serum (FCS; Paesel, Frankfurt, Germany) or various concentrations of autologous plasma, respectively. Single growth factors or growth factor combinations were added immediately after seeding the $CD34^+$ cells into the microtiter plates (total volume, 200 μL/well). Quadruplicate cultures of each of the 36 growth factor combinations tested were established. The following hematopoietic growth factors and cytokines were used: IL-1, IL-3, granulocyte-macrophage–CSF (GM-CSF) (all provided by Behringwerke, Marburg, Germany), IL-6 (Boehringer Mannheim, Mannheim, Germany), macrophage-CSF (M-CSF), leukemia inhibitory factor (LIF) (kindly provided by K. Welte, Hannover, Germany), interferon-γ (IFN-γ; Polyferon, Thomae, Germany), and SCF (Amgen, Thousand Oaks, CA). Growth factors were used at a concentration of 100 ng/mL (IL-1, G-CSF, GM-CSF, M-CSF, IFN-γ, and SCF), 100 U/mL (IL-3 and IL-6), or 10 ng/mL (LIF), respectively. Erythropoietin (EPO; Cilag, Bad Homburg, Germany) was used at 1 U/mL. Cells were incubated for up to 28 days at 37°C in 5% CO_2 without additional feeding of growth factors or medium. For analysis, each well was resuspended and washed in RPMI 1640 to remove residual growth factors. Viability of cells was assessed by trypan blue dye exclusion as well as by flow cytometric staining with propidium iodide (5 μg/mL). Morphology of Giemsa-stained cells was performed on cytospin preparations.

Mafosfamide (4-hydroperoxycyclophosphamide [4-HC]; ASTA Medica, Frankfurt, Germany) treatment of $CD34^+$ cells was performed as described.[23] Briefly, $CD34^+$ cells were incubated with 4-HC (30 μg/mL) at 37°C for 45 minutes washed twice in RPMI 1640 medium, and cultured in suspension up to 24 days as described above.

The number of expanded nucleated cells as well as their colony-forming capacity (see below) was analyzed for each growth factor combination.

Large-scale expansion of enriched $CD34^+$ cells. Scaled-up expansion of enriched $CD34^+$ cells from the leukapheresis products of two different patients (1 patient with limited-disease small cell lung cancer and 1 patient with a refractory non-Hodgkin's lymphoma) were performed in 250 mL tissue culture flasks (Falcon) in 1% heparinized autologous plasma. A 100 mL culture of $CD34^+$ cells was established at 1.5×10^4 $CD34^+$ cells/mL. The cells were incubated for 21 days without further addition of any growth factors. Serial samples of cells were removed at different time points and analyzed with respect to morphology, immunophenotype, and colony-forming capacity.

Clonogenic assays for myeloid (colony-forming unit–granulocyte-macrophage [CFU-GM]), erythroid (burst-forming unit-erythroid [BFU-E]), and multilineage (CFU-granulocyte, erythrocyte, monocyte, megakaryocyte [CFU-GEMM]) progenitors. Unseparated MNCs from the leukapheresis product, enriched $CD34^+$ cells, as well as expanded cells were grown in 0.9% methylcellulose as described.[24] Unseparated MNCs were cultured at 1×10^5/mL in 30% FCS. Positively enriched $CD34^+$ cells as well as expanded cells were cultured at three different cell concentrations (5×10^3/mL, 1.5×10^4/mL, and 5×10^4/mL). The assay was found to be linear up to a seeded cell number of 1.5×10^4/mL. All semisolid cultures were performed in duplicate and stimulated with 100 ng/mL SCF, 100 ng/mL IL-1β, 100 ng/mL GM-CSF, 20 U/mL IL-3, 100 U/mL IL-6, and EPO (1 U/mL).

The absolute number of CFU-GM, BFU-E, and CFU-GEMM grown at each time point during the liquid culture was calculated by multiplying their incidence (per cells seeded in methylcellulose) by the number of living nucleated cells present in each liquid culture.

Dual-color or triple-color flow cytometry. PB MNCs, enriched $CD34^+$ cells, as well as expanded nucleated cells from suspension cultures were incubated with anti-CD34 fluorescein (FITC)-conjugated or phycoerythrin (PE)-conjugated MoAbs (HPCA-2; Becton Dickinson, Rödermark, Germany) and/or anti-CD33–PE, anti-CD38–PE, anti-CD14–FITC, anti-CD11b–FITC, anti-CD15–FITC, anti-CD3–PerCP, or anti-HLA-DR–PerCP conjugated MoAbs (all from Becton Dickinson) for 30 minutes at 4°C. Cells were analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson) equipped with a filter set for FITC-PE dual-color fluorescence. Data acquisition was performed with FACScan Lysis II research software. Each measurement included 30,000 cells.

Immunocytology of expanded cells. Enriched $CD34^+$ cells as well as expanded nucleated cells were attached to alcian blue-coated slides and fixed in 0.05% glutaraldehyde as described.[25,26] For analysis, the following mouse MoAbs were used: anti-CD34 (HPCA-1; Becton Dickinson), anti-CD33 (My 9; Coulter, Krefeld, Germany), anti-HLA-DR, and anti-CD38 (all from Becton Dickinson). A peroxidase-antiperoxidase (PAP) technique was applied, followed by postfixation with OsO_4.

Statistics. The statistical significance of the data obtained were analyzed by the Wilcoxon's rank sum test and by the Student's *t*-test. A P value $<.05$ was considered significant.

RESULTS

Characterization of enriched $CD34^+$ cells before expansion in suspension culture. Harvested, VIP + G-CSF recruited PBPCs contained a median of 2.4% $CD34^+$ cells (range, 0.8% to 6.3%), as determined by flow cytometry. The purity of positively selected $CD34^+$ cells after immunoadsorption was 87% (range, 69% to 97%) (Fig 1). $CD34^+$ cells were blast-like cells with typical small basophilic cytoplasm. Contaminating cells included granulocytes and less than 3% T lymphocytes or monocytes. The viability of $CD34^+$ cells was more than 95%. Enriched $CD34^+$ cells coexpressed CD38 (median, 98%; range, 94% to 100%) and HLA-DR (median, 93%; range, 86% to 100%), whereas CD33 coexpression was variable (median, 40%; range, 23% to 84%). $CD34^+/CD38^-$ cells were present at a median of 1.1% (range, 0.3% to 1.8%); $CD34^+/HLA\text{-}DR^-$ cells represented 4.6% (range, 1.6% to 13%).

In vitro expansion of enriched $CD34^+$ cells. A total of 36 hematopoietic growth factor combinations was tested to investigate the expansion of PB $CD34^+$ cells in liquid culture. Maximal expansion of both total nucleated cells (median, 260-fold increase; range, 76- to 995-fold) as well as clonogenic progenitor cells (median, 190-fold increase; range, 46- to 930-fold) was observed in the presence of SCF, Epo, IL-1, IL-3, and IL-6 (Fig 2). The patient-to-patient variation was considerably high, depending on the prior treatment status of the patients as well as the purity of enriched $CD34^+$ cells; in 4 patients using non–column-separated PBPCs, expansion of clonogenic progenitor cells was significantly less ef-

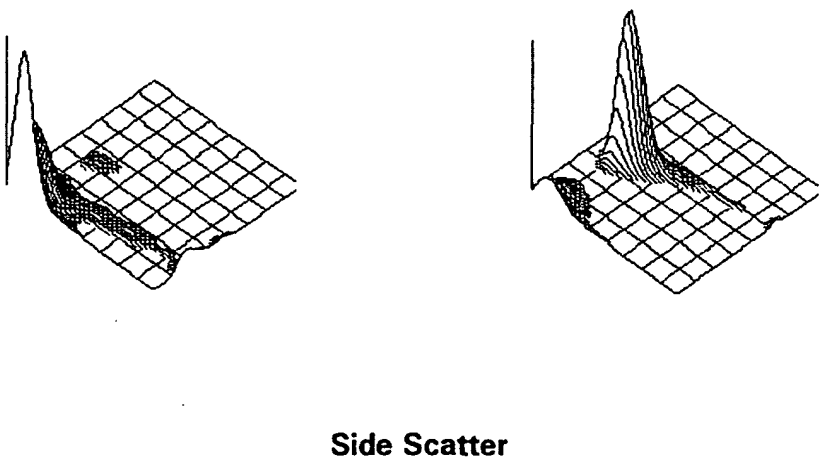

Fig 1. Flow cytometry analysis of PB CD34+ cells before and after positive selection by immunoadsorption (Three-dimensional contour plot of one representative patient; right-angle scatter v HPCA-2–PE fluorescence). CD34+ cells were enriched to a median of 87% (range, 69% to 97%) after column separation.

fective as compared with column-separated cells (median, 38-fold increase; range, 5- to 143-fold). Single growth factors as well as several combinations of two factors (eg, SCF + IL-3) were significantly less effective in expanding progenitors when compared with 3-, 4-, or 5-factor combinations. Selective removal of either SCF or Epo from the optimal 5-factor combination resulted in significantly lower numbers of expanded clonogenic progenitor cells (Fig 2). The removal of IL-1, IL-3, or IL-6 from SCF and Epo-containing media also resulted in a reduced number of progenitor cells (Fig 2). The further addition of G-CSF or M-CSF to the optimal 5-factor combination did not show synergistic effects. Interestingly, although the addition of GM-CSF or LIF to the optimal 5-factor combination resulted in an even higher expansion of total nucleated cells, the median number of colony-forming cells was significantly decreased (GM-CSF: median, 63-fold increase; range, 14- to 339-fold) (Figs 2 and 3). However, the addition of IFN-γ (100 U/mL) synergistically enhanced the generation of both nucleated cells (median, 390-fold; range, 120- to 870-fold) as well as clonogenic

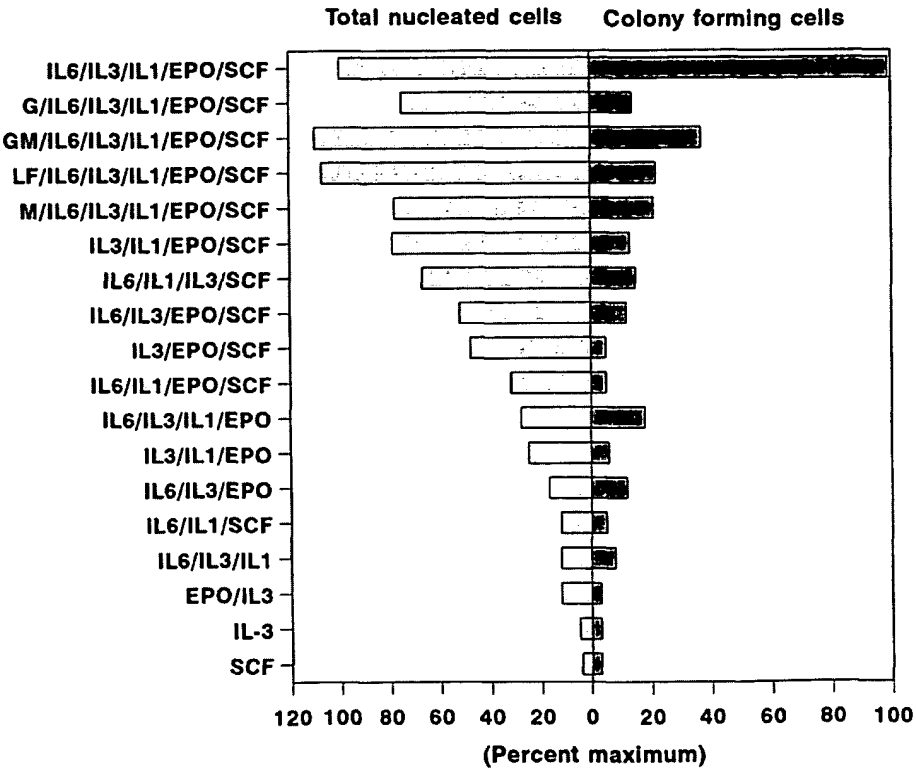

Fig 2. Effect of hematopoietic growth factor combinations on the ex vivo expansion of PB CD34+ cells. Data are presented as the median percentage of the maximum levels of total nucleated cells (left panel) and total clonogenic progenitor cells (right panel) for several combinations of growth factors, as compared with the optimal 5-factor combination with SCF, IL-1, IL-3, IL-6, and EPO.

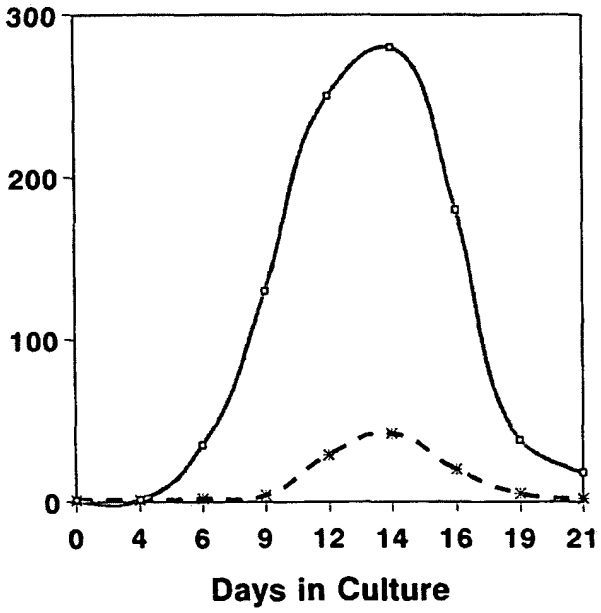

Fig 3. Kinetic analysis of the ex vivo expansion of CD34+ cells induced by SCF, IL-1, IL-3, IL-6, and EPO with GM-CSF (*) or without GM-CSF (□). Data are presented as the fold increase of colony-forming cells from one patient.

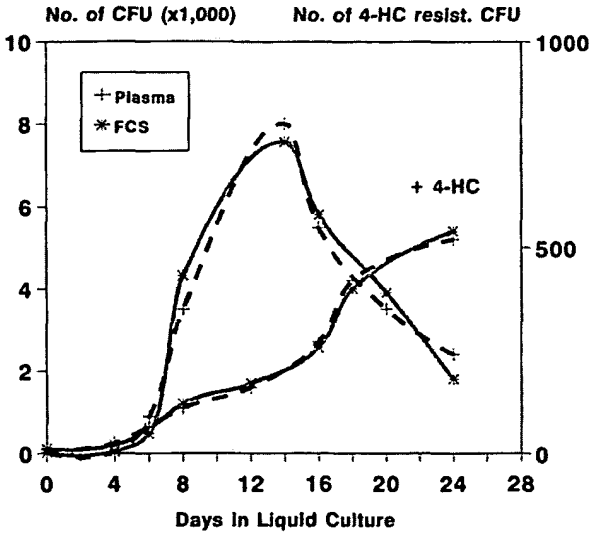

Fig 5. Kinetic studies of the expansion of CD34+ cells in the presence of SCF, IL-1, IL-3, IL-6, and EPO with or without mafosfamide (4-HC) pretreatment. Data are presented as the number of total clonogenic progenitor cells per culture in the presence of 1% autologous plasma or 10% FCS.

progenitor cells (median, 260-fold; range, 81- to 760-fold) (Fig 4).

Kinetic studies showed a continuous increase in total cell numbers until day 16, followed by a decline of total cell numbers until day 24. The number of clonogenic progenitor cells of different lineages started to increase at day 6 and peaked at days 12 to 14 in culture (Fig 5). Mafosfamide-resistant CD34+ cells started to proliferate significantly later as compared with unmanipulated CD34+ cells and continuously proliferated at least until day 24 in culture (Fig 5).

Comparison of FCS versus autologous plasma-containing cultures showed that a concentration of 1% autologous plasma was as effective as a concentration of 10% FCS (Fig 5). Therefore, the subsequent large-scale analyses were performed in the presence of 1% autologous plasma plus the optimal 5-factor combination of hematopoietic growth factors.

Analyses of different subtypes of clonogenic progenitors showed an increase in myeloid (median, 190-fold; range, 18- to 353-fold), erythroid (median, 236-fold; 21- to 514-fold), and multilineage (median, 250-fold; range, 33- to 589-fold) colonies when compared with pre-expansion values (Fig 6).

Immunophenotype of expanded CD34+ cells. The number of CD34+ cells during expansion rapidly decreased to a

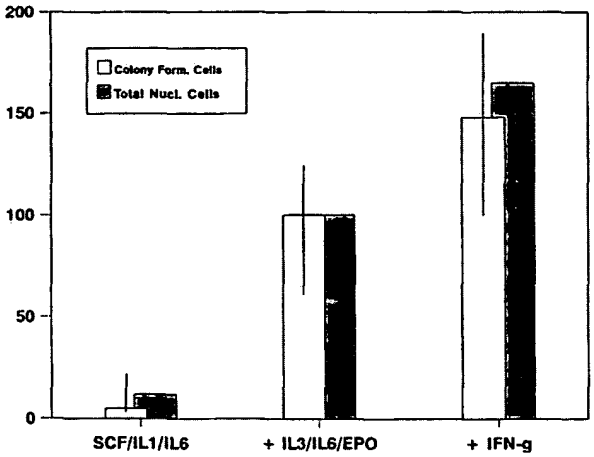

Fig 4. Effect of IFN-γ on the expansion of PBPCs in SCF, IL-1, IL-3, IL-6, and EPO containing cultures. Data are presented as median fold increase of colony-forming cells or total nucleated cells.

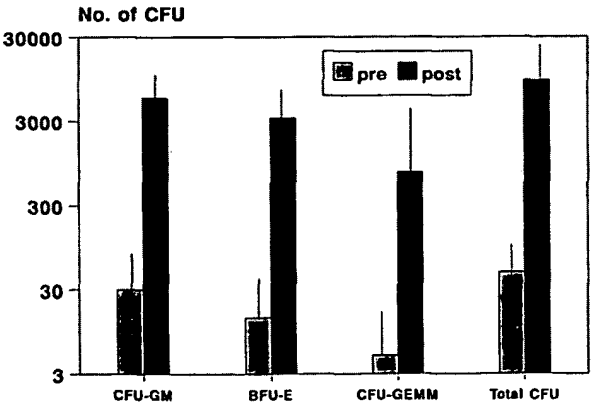

Fig 6. Analyses of subtypes of clonogenic progenitors after in vitro expansion of CD34+ cells by SCF, IL-1, IL-3, IL-6, and EPO. Data are presented as the median absolute number of CFU-GM, BFU-E, or CFU-GEMM colonies per culture before and after ex vivo expansion.

median of 1.5% (range, 0.2% to 2.5%) at day 14 in culture. However, the absolute number of $CD34^+$ cells present in each well increased threefold (range, 1- to 12-fold) to approximately 1×10^4 $CD34^+$/well at the day of maximal proliferation. The percentage of $CD34^+/CD38^-$ cells (range, 3% to 14%) or $CD34^+/HLA\text{-}DR^-$ cells (range, 7% to 28%), as well as the absolute number of lineage-negative $CD34^+$ cells, increased ninefold (range, 3- to 21-fold) as compared with pre-expansion values. Interestingly, the number of $CD34^+/HLA\text{-}DR^-$ progenitor cells was found to be synergistically increased by the addition of IFN-γ to the optimal 5-factor combination (median, 24%; range, 11% to 39%).

Large-scale expansion of enriched $CD34^+$ cells in SCF, IL-1, IL-6, IL-3, and EPO. Enriched $CD34^+$ cells (1.5×10^6) from two patients were expanded in a 250 mL tissue culture flask containing 1% autologous plasma and SCF, IL-1, IL-3, IL-6, and EPO. Cells were shown to proliferate in a similar fashion as compared with the microwell system. The percentage of $CD34^+$ cells rapidly decreased, whereas the number of clonogenic progenitor cells increased 130-fold and 210-fold, respectively, at day 14 of culture. Expansion of total nucleated cells was 170- and 240-fold, respectively.

DISCUSSION

This study documents the expansion of PB $CD34^+$ cells ex vivo by a combination of 5 hematopoietic growth factors, ie, SCF, IL-1, IL-3, IL-6, and EPO. The median increase in clonogenic progenitor cells of different hematopoietic lineages (CFU-GM, BFU-E, and CFU-GEMM) was 190-fold at day 14 during suspension culture. The addition of IFN-γ to this 5-factor combination was shown to further increase the number of clonogenic cells by a factor of 2. The effect of IFN-γ is in accordance with a previous report showing an enhancing effect of IFN-γ on the frequency of growth factor-responding cells.[27] Although the number of clonogenic progenitor cells increased 190-fold, the absolute number of $CD34^+$ cells increased only up to 12-fold during ex vivo expansion. These data indicate that the cloning efficiency of $CD34^+$ cells after ex vivo culture increased as compared with that of initially seeded $CD34^+$ cells. Alternatively, an alteration of the density or glycosylation pattern of the CD34 antigen on the progenitor cells during ex vivo culture might interfere with its detection by immunophenotyping.

Large-scale expansion of $CD34^+$ cells in autologous plasma supplemented with SCF, IL-1, IL-3, IL-6, and EPO was feasible without additional feeding and was shown to have a similar time course and a similar increase in the generation of clonogenic progenitor cells as compared with the microculture system. Recently, a pilot study on the large-scale expansion of FACS-sorted PB $CD34^+$ cells from three patients was published by Haylock et al.[28] The investigators showed the feasibility of such an approach with a 6-factor combination including SCF, IL-1, IL-3, IL-6, G-CSF, and GM-CSF. The expansion in this system generated predominantly myeloid precursor cells at various stages of differentiation, with a median 66-fold increase in CFU-GM progenitors.[28] Therefore, the investigators hypothesize that the clinical use of ex vivo expanded postprogenitor cells with the simultaneous retransfusion of nonexpanded PBPCs possibly further shortens the rate of hematopoietic reconstitution after PB stem cell transplantation.

Interestingly, the addition of G-CSF or GM-CSF to our liquid culture system significantly reduced the colony formation, probably due to the predominant induction of differentiation, producing large numbers of terminally differentiated neutrophil precursors without clonogenic capacity. Because the aim of our study was the ex vivo production of clonogenic progenitor cells for repetitive use after high-dose chemotherapy, we tried to expand $CD34^+$ cells ex vivo by using "early acting" hematopoietic growth factors to preserve the capacity for multilineage colony formation.

FACS sorting of pre-enriched $CD34^+$ cells (as performed by Haylock et al[28]) for the initiation of large-scale ex vivo cultures would be a time-consuming procedure and might hamper its clinical application. Thus, the use of a device for the rapid selection of large quantities of $CD34^+$ cells from leukapheresis products or blood samples would be requested. We used an avidin-biotin immunoaffinity column, which was shown to be effective in the positive selection of $CD34^+$ cells, both from marrow as well as from PB.[29,30]

Assuming a threshold dose of 3×10^7 CFU-GM for successful engraftment in an adult human[31] and an average 260-fold expansion of CFU-GM progenitors, a starting cell number of 1.2×10^5 CFU-GM would be necessary for autotransplantation. Considering a median of 2.4% $CD34^+$ cells within the leukapheresis product with a cloning efficiency of 2% and a 50% loss of these cells upon immunoadsorption, 6×10^6 $CD34^+$ cells from the patient would be necessary, being equivalent to 5% of the total $CD34^+$ cells present within one single leukapheresis. Alternatively, from a theoretical point of view, a blood sample of 350 mL would be sufficient to isolate 6×10^6 $CD34^+$ cells by immunoadsorption, considering 1.5×10^6 mononuclear cells/mL blood at the time of maximal levels of $CD34^+$ cells upon mobilization with VIP chemotherapy plus G-CSF,[20] which corresponds to a total of 3.6×10^4 $CD34^+$ cells/mL of whole blood. This actually would mean that apheresis procedures could be avoided if only 350 mL of whole blood need to be drawn, purified, and expanded ex vivo for successful autotransplantation. However, such calculations assume the functional in vivo integrity of expanded PBPCs as to short- and long-term engraftment, an implication that has not yet been proven.

The majority of clonogenic cells generated in our system are myeloid, erythroid, and multilineage progenitor cells. However, ex vivo expanded cells also include early hematopoietic progenitor cells as analyzed by the presence of $CD34^+/HLA\text{-}DR^-$ or $CD38^-$ cells as well as 4-HC–resistant colony-forming cells. Because in vivo models for long-term repopulating capability with human hematopoietic progenitor cells are not available, with the exception of the SCID-hu mouse system,[32-34] clinical trials in patients with advanced malignancies will be necessary to show if ex vivo expanded PB $CD34^+$ progenitor cells would be equivalent to the biologic capabilities of unmodified PBPCs.

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